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 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
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Date of mailing (day/month/year) 15 March 2001 (15.03.01)	
International application No. PCT/KR00/00733	Applicant's or agent's file reference PCA00729/HMY
International filing date (day/month/year) 07 July 2000 (07.07.00)	Priority date (day/month/year) 08 July 1999 (08.07.99)
Applicant KWON, Se, Chang et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

01 February 2001 (01.02.01)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Pascal Piriou Telephone No.: (41-22) 338.83.38
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/04329 A1

(51) International Patent Classification⁷: **C12N 15/70**

(21) International Application Number: **PCT/KR00/00733**

(22) International Filing Date: **7 July 2000 (07.07.2000)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:
1999/27418 ✓ **8 July 1999 (08.07.1999) KR**

(71) Applicant (for all designated States except US): **HANMI PHARM. CO., LTD. [KR/KR]; 893-5, Haejeon-ri, Paltan-myeon, Hwasong-gun, Kyungki-do 445-910 (KR).**

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KWON, Se, Chang [KR/KR]; Hanyang Apt. 5-201, 789, Siheung-1-dong, Kumcheon-gu, Seoul 153-031 (KR). JUNG, Sung, Youb [KR/KR]; Keoyeo Apt. 504-1402, 294, Keoyeo-2-dong,**

Songpa-gu, Seoul 138-112 (KR). **BAE, Sung, Min [KR/KR]; 1587-8, Bongchun-4-dong, Kwanak-gu, Seoul 151-054 (KR). LEE, Gwan, Sun [KR/KR]; Keukdong Apt. 2-806, Karak-dong, Songpa-gu, Seoul 138-160 (KR).**

(74) Agents: **JANG, Seong, Ku et al.; KEC Building, 17th floor, 275-7, Yangjae-dong, Seocho-ku, Seoul 137-130 (KR).**

(81) Designated States (national): **AU, BR, CA, CN, JP, NZ, RU, SG, US.**

(84) Designated States (regional): **European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).**

Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **MODIFIED HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR AND PROCESS FOR PRODUCING SAME**

T P L G P A S S L P Q S F L L K
aca ccc ctg ggc cct gcc agc tcc ctg ccc cag agc ttc ctg ctc aag
C L E Q V R K I Q G D G A A L Q
tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag
E K L C A T Y K L C H P E E L V
gag aag ctg tgt gcc acc tac aag ctg tgc cac ccc gag gag ctg gtg
L L G H S L G I P W A P L S S C
ctg ctc gga cac tct ctg gcc atc ccc tgg gct ccc ctg agc tcc tgc
P S Q A L Q L A G C L S Q L H S
ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa ctc cat agc
G L F L Y Q G L L Q A L E G I S
ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa ggg ata tcc
P E L G P T L D T L Q L D V A D
ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac gtc gcc gac
F A T T I W Q Q M E E L G M A P
ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga atg gcc cct
A L Q P T Q G A M P A F A S A F
gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc tct gct ttc
Q R R A G G V L V A S H L Q S F
cag cgc cgg gca gga ggg gtc ctg gtt gct agc cat ctg cag agc ttc
L E V S Y R V L R H L A Q P
ctg gag gtg tgg tac cgc gtt cta cgc cac ctt gcg cag ccc

(57) Abstract: A modified human granulocyte-colony stimulating factor (hG-CSF) is produced by culturing a microorganism transformed with an expression vector comprising a gene encoding a modified hG-CSF to produce and secrete the modified hG-CSF to periplasm, said modified hG-CSF being obtained by replacing at least one of the 1st, 2nd, 3rd and 17th amino acids of wild-type hG-CSF (SEQ ID NO: 2) with other amino acid.

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MODIFIED HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR AND PROCESS FOR PRODUCING SAME

Field of the Invention

The present invention relates to a modified human granulocyte-colony stimulating factor(hG-CSF), a gene encoding said peptide, a vector comprising said gene, a microorganism transformed with said vector and a process for producing the modified hG-CSF using said microorganism.

Background of the Invention

The term colony stimulating factor (CSF) is inclusive of granulocyte/macrophage-colony stimulating factor(GM-CSF), macrophage-colony stimulating factor(M-CSF) and granulocyte-colony stimulating factor(G-CSF), which are produced by T-cells, macrophages, fibroblasts and endothelial cells. GM-CSF stimulates stem cells of granulocyte or macrophage to induce the differentiation thereof and proliferation of granulocyte or macrophage colonies. M-CSF and G-CSF primarily induce the formation of the colonies of macrophage and granulocyte, respectively. In vivo, G-CSF induces the differentiation of bone marrow leucocytes and enhances the function of mature granulocyte and, accordingly, it's clinical importance in treating leukemia has been well established.

Human G-CSF(hG-CSF) is a protein consisting of 174 or 177 amino acids, the 174 amino-acid variety having higher neutrophil-enhancing activity(Morishita, K. et al., *J. Biol. Chem.*, 262, 15208-15213(1987)). The amino acid sequence of hG-CSF consisting of 174 amino acids is shown in Fig. 1 and there have been many studies for the mass production of hG-CSF by manipulating a gene encoding said hG-CSF.

For instance, Chugai Pharmaceuticals Co., Ltd.(Japan) has disclosed the amino acid sequence of hG-CSF and a gene encoding same(Korean Patent Publication Nos. 91-5624 and 92-2312), and reported a method for preparing proteins having hG-CSF activity by a gene recombination process(Korean Patent Nos. 47178, 53723 and 57582). In this preparation method, glycosylated hG-CSF is produced in a mammalian cell by employing a genomic DNA or cDNA comprising a polynucleotide encoding hG-CSF. The glycosylated hG-

CSF has an O-glycosidic sugar chain, but, it is known that said sugar chain is not necessary for the activity of hG-CSF(Lawrence, M. et al., Science, 232, 61(1986)). Further, it is also well-known that the production of glycosylated hG-CSF employing mammalian cells requires expensive materials and facilities, and therefore, such a process is not economically feasible.

Meanwhile, there have been attempts to produce non-glycosylated hG-CSF by employing a microorganism, e.g., *E. coli*. In these studies, hG-CSFs having 175 or 178 amino acids having a methionine residue attached at the N-terminus thereof are obtained due to the ATG initiation codon employed in the microorganism. The additional methionine residue, however, causes undesirable immune responses in human body when the recombinant hG-CSF is administered thereto(European Patent Publication No. 256,843). Further, most of the methionine-containing hG-CSFs produced in *E. coli* are deposited in the cells as insoluble inclusion bodies, and they must be converted to an active form through a refolding process, at a significant loss of yield. In this regard, four of the five Cys residues present in wild-type hG-CSF participate in forming disulfide bonds, while the remaining one contributes to the aggregation of the hG-CSF product during the refolding process to lower the yield.

Recently, in order to solve the problems associated with the production of a foreign protein within a microbial cell, various efforts have been made to develop a method based on efficient secretion of a target protein across the microbial cell membrane into the extra-cellular domain.

For instance, in a method employing a signal peptide, a desired protein is expressed in the form of a fusion protein wherein a signal peptide is added to the N-terminus of the protein. When the fusion protein passes through the cell membrane, the signal peptide is removed by an enzyme and the desired protein is secreted in a mature form. The secretory production method is advantageous in that the produced amino acid sequence is usually identical to the wild-type. However, the yield of a secretory production method is often quite low due to unsatisfactory efficiencies in both the membrane transport and the subsequent purification process. This is in line with the well-known fact that the yield of a mammalian protein produced in a secretory mode in prokaryotes is very low: Hitherto, no microbial method has been reported for the efficient expression and secretion of soluble hG-CSF having no added methionine residue at its N-terminus.

The present inventors have previously reported the use of a new

secretory signal peptide prepared by modifying the signal peptide of *E. coli* thermoresistant enterotoxin II(Korean Patent Laid-open publication No. 2000-19788) in the production of hG-CSF. Specifically, an expression vector comprising a hG-CSF gene attached to the 3'-end of the modified signal peptide of *E. coli* thermoresistant enterotoxin II was prepared, and biologically active, mature hG-CSF was expressed by employing *E. coli* transformed with the expression vector. However, most of the expressed hG-CSF accumulated in the cytoplasm rather than in the periplasm.

The present inventors have endeavored further to develop an efficient secretory method for the production of hG-CSF in a microorganism and have found that a modified hG-CSF, which is prepared by replacing at least one amino acid residue, especially, the 17th cysteine residue, of wild-type hG-CSF with other amino acid, retains the biological activity of the wild-type, and that the modified hG-CSF having no methionine residue at the N-terminus thereof can be efficiently expressed and secreted by a microorganism when an appropriate secretory signal peptide is employed.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a modified human granulocyte-stimulating factor(hG-CSF) which can be efficiently produced using a microorganism..

It is another object of the present invention to provide a gene encoding said peptide and a vector comprising said gene.

It is a further object of the present invention to provide a microorganism transformed with said vector.

It is a still further object of the present invention to provide a process for producing a hG-CGF which is non-attached methionine residue to amino terminus using said microorganism.

In accordance with one aspect of the present invention, there is provided a modified hG-CSF characterized in that at least one of the 1st, 2nd, 3rd and 17th amino acids of wild-type hG-CSF is replaced by another amino acid.

Brief Description of the Drawings

5 The above and other objects and features of the present invention will become apparent from the following description of the invention taken in conjunction with the following accompanying drawings; which respectively show:

Fig.1 : the nucleotide and amino acid sequences of wild-type human granulocyte-stimulating factor composed of 174 amino acids residues(SEQ ID NOS: 1 and 2);

Fig. 2 : the procedure for constructing vector pT-CSF;

Fig. 3 : the procedure for constructing vector pT14S1SG;

Fig. 4 : the procedure for constructing vector pT14SS1SG;

Fig. 5 : the procedure for constructing vector pT140SSG-4T22Q;

15 Fig. 6 : the procedure for constructing vector pT14SS1S17SEG;

Fig. 7 : the procedure for constructing vector pTO1SG;

Fig. 8 : the procedure for constructing vector pBADG;

Fig. 9 : the procedure for constructing vector pBAD2M3VG;

20 Figs. 10a and 10b : the results of western blot analyses which verify the expression of hG-CSF and modified hG-CSFs from recombinant cell lines and the molecular weight of expressed proteins, respectively; and

Fig. 11 : the cellular activities of hG-CSF and modified hG-CSF produced from recombinant cell lines.

Detailed Description of the Invention

The modified hG-CSFs of present invention are derived by replacing one or more of the amino acids of wild-type hG-CSF(SEQ ID NO: 2), preferably the 1st, 2nd, 3rd and 17th amino acids thereof, by other amino acids. More preferred are those obtained by replacing the 17th amino acid of hG-CSF with an amino acid which is uncharged at neutral pH. Specific examples of preferred modified hG-CSFs have the amino acid sequence of wild-type hG-CSF, except that:

- 35 (a) the 1st amino acid is Ser;
- (b) the 1st amino acid is Ser and the 17th amino acid is X;
- (c) the 2nd amino acid is Met and the 3rd amino acid is Val;

(d) the 2nd amino acid is Met, the 3rd amino acid is Val and the 17th amino acid is X; or

(f) the 17th amino acid is X,

wherein X is an amino acid which is not charged at neutral pH.,
5 preferably Ser, Thr, Ala or Gly, more preferably Ser.

Four of the five Cys residues of hG-CSF participate in forming disulfide bonds, while the 17th Cys residue remains unbonded in the natural state. However, when a large amount of hG-CSF is expressed in recombinant
10 cells, the 17th Cys residue gets involved in inter-molecular disulfide bond formation, leading to the accumulation of agglomerated hG-CSFs in the cytoplasm. However, the inventive modified hG-CSF having an amino acid other than Cys at the 17th position is free of such problem and can be effectively produced by a secretory method using an appropriately transformed
15 microorganism.

The modified hG-CSF of the present invention may be encoded by a gene comprising a nucleotide sequence deduced from the modified hG-CSF amino acid sequence according to the genetic code. It is known that several different codons encoding a specific amino acid may exist due to the codon
20 degeneracy, and, therefore, the present invention includes in its scope all nucleotide sequences deduced from the modified hG-CSF amino acid sequence. Preferably, the modified hG-CSF gene sequence includes one or more preferred codons of *E. coli*.

The gene thus prepared may be inserted to a conventional vector to
25 obtain an expression vector, which may, in turn, be introduced into a suitable host, e.g., an *E. coli*. The expression vector may further comprise a signal peptide. Representative signal peptides include a thermoresistant *E. coli* enterotoxin II signal peptide(SEQ ID NO: 53), a modified thermoresistant *E. coli* enterotoxin II signal peptide(SEQ ID NO: 54), a beta lactamase signal
30 peptide(SEQ ID NO: 24), Gene III signal peptide(SEQ ID NO: 42) or modified peptide thereof, but these do not limit the signal peptides which may be used in the present invention. The promoter used in preparing the expression vector of present invention may be any of those which can express a heterologous protein in a microorganism host. Specially, lac, Tac, and arabinose promoter is
35 preferred when the heterologous protein is expressed from *E. coli*.

Exemplary expression vector of the present invention includes

pT14SS1SG, pT14SS1S17SEG, pTO1SG, pTO1S17SG, pTO17SG, pTO17TG, pTO17AG, pTO17GG, pBAD2M3VG, pBAD17SG and pBAD2M3V17SG.

The expression vectors of the present invention may be introduced into microorganism, e.g., *E. coli* BL21(DE3)(Novagen), *E. coli* XL-1 blue(Novagen) according to a conventional transformation method(Sambrook et al., the supra) to obtain transformants *E. coli* BL21(DE3)/pT14SS1SG(HM 10310), *E. coli* BL21(DE3)/pT14SS1S17SEG(HM 10311), *E. coli* BL21(DE3)/pTO1SG(HM 10409), *E. coli* BL21(DE3)/pTO1S17SG(HM 10410), *E. coli* BL21(DE3)/pTO17SG(HM 10411), *E. coli* BL21(DE3)/pTO17TG(HM 10413), *E. coli* BL21(DE3)/pTO17AG(HM 10414), *E. coli* BL21(DE3)/pTO17GG(HM 10415), *E. coli* BL21(DE3)/pBAD2M3VG(HM 10510), *E. coli* BL21(DE3)/pBAD17SG(HM 10511) and *E. coli* BL21(DE3)/pBAD2M3V17SG(HM 10512). Among the transformed microorganism, preferred are transformants *E. coli* BL21(DE3)/pT14SS1S17SEG(HM 10311), *E. coli* BL21(DE3)/pTO1S17SG(HM 10410), *E. coli* BL21(DE3)/pTO17SG(HM 10411) and *E. coli* BL21(DE3)/pBAD2M3VG(HM 10510) which were deposited with Korean Culture Center of Microorganisms(KCCM)(Address; Department of Food Engineering, College of Eng., Yonsei University, Sodaemun-gu, Seoul 120-749, Republic of Korea) on March 24, 1999 under accession numbers KCCM-10154, KCCM-10151, KCCM-10152 and KCCM-10153, respectively, in accordance with the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

The modified hG-CSF protein of the present invention may be produced by culturing the transformant microorganism to express the gene encoding the modified hG-CSF protein and secrete the modified hG-CSF, protein to periplasm; and recovering the modified hG-CSF protein from the periplasm. The transformant microorganism may be cultured in accordance with a conventional method(Sambrook et al., the supra). The microorganism culture may be centrifuged or filtered to collect the microorganism secreting the modified hG-CSF protein. The transformed microorganism may be disrupted according to a conventional method(Ausubel, F. M. et al., Current Protocols in Molecular Biology, (1989)) to obtain a periplasmic solution. For example, the microorganism may be disrupted in a hypotonic solution, e.g., distilled water, by an osmotic shock. Recovery of the modified hG-CSF in the periplasmic

solution may be conducted by a conventional method(Sambrook et al., the supra), e.g., ion exchange chromatography, gel filtration column chromatography or immune column chromatography. For example, hG-CSF may be purified by sequentially conducting CM-Sepharose column chromatography and Phenyl Sepharose column chromatography.

The modified hG-CSF protein produced according to the present invention is not methionylated at the N-terminus and has biological activity which is equal to, or higher than, that of wild-type hG-CSF. Therefore, it may be used as is in various applications

The following Examples are intended to further illustrate the present invention without limiting its scope.

Example 1: Preparation of A Gene Encoding hG-CSF

A cDNA gene encoding hG-CSF was prepared by carrying out PCR using as an hG-CSF template(R&D system, USA). The primers used are those described in US patent No. 4,810,643.

To prepare a cDNA gene encoding mature hG-CSF, vector pUC19-G-CSF(Biolabs, USA) was subjected to PCR using the primers of SEQ ID NOS: 3 and 4. The primer of SEQ ID NO: 3 was designed to provide an NdeI restriction site(5'-CATATG-3') upstream from the first amino acid(threonine) codon of mature hG-CSF, and the primer of SEQ ID NO: 4, to provide a BamHI restriction site(5'-GGATCC-3') downstream from the termination codon thereof.

The amplified hG-CSF gene was cleaved with NdeI and BamHI to obtain a gene encoding mature hG-CSF. The hG-CSF gene was inserted at the NdeI/BamHI section of vector pET14b(Novagen, USA) to obtain vector pT-CSF.

Fig. 2 shows the above procedure for constructing vector pT-CSF.

Example 2: Construction of a vector containing the gene encoding *E. coli* enterotoxin II signal peptide and a modified hG-CSF

(Step 1) Cloning *E. coli* enterotoxin II signal peptide gene

To prepare *E. coli* enterotoxin II signal peptide gene, the pair of complementary oligonucleotides having SEQ ID NOS: 5 and 6 were designed

based on the nucleotide sequence of *E. coli* enterotoxin II signal peptide, and synthesized using DNA synthesizer(Model 380B, Applied Biosystem, USA).

The above oligonucleotides were designed to provide BspHI restriction site(complementary sites to an NcoI restriction sites) upstream from the initiation codon of *E. coli* enterotoxin II and an MluI restriction site introduced by a silent change at the other end.

Both oligonucleotides were annealed at 95 °C to obtain blunt-ended DNA fragments having a nucleotide sequence encoding *E. coli* enterotoxin II signal peptide(STII gene).

The STII gene was inserted at the SmaI site of vector pUC19(Biolabs, USA) to obtain vector pUC19ST.

(Step 2) Preparation of a gene encoding STII/hG-CSF

To prepare a gene encoding STII/hG-CSF, vector pT-CSF obtained in Preparation Example 1 was subjected to PCR using the primers of SEQ ID NOS: 7 and 8. The primer of SEQ ID NO: 7 was designed to substitute Ser codon for the first codon of hG-CSF, and the primer of SEQ ID NO: 8, to provide a BamHI restriction site(5'-GGATCC-3') downstream from the termination codon thereof.

The amplified DNA fragments were cleaved with MluI and BamHI, and then inserted at the MluI/BamHI section of pUC19ST obtained in Step 1 to obtain vector pUC19S1SG. Vector pUC19S1SG thus obtained contained a gene encoding an STII/hG-CSF(designated STII-hG-CSF gene).

Vector pUC19S1SG was cleaved with BspHI and BamHI to obtain a DNA fragment(522 bp). The DNA fragment was inserted at the NcoI/BamHI section of vector pET14b(Novagen, USA) to obtain vector pT14S1SG.

Fig. 3 depicts the above procedure for constructing vector pT14S1SG.

(Step 3) Addition of *E. coli* enterotoxin II Shine-Dalgarno sequence to STII-hG-CSF gene

Vector pT14S1SG obtained in Step 2 was subjected to PCR using the primers of SEQ ID NOS: 9 and 10. The primer of SEQ ID NO: 9 was designed to provide an *E. coli* enterotoxin II Shine-Dalgarno sequence(designated STII SD sequence) and an XbaI restriction site, and the

primer of SEQ ID NO: 10, to provide a BamHI restriction site downstream from the termination codon of mature hG-CSF to obtain a DNA fragment(STII SD-STII-hCSF) containing a STII SD and STII-hG-CSF gene.

5 The STII SD-STII-hG-CSF fragment was cleaved with XbaI and BamHI, and then inserted at the XbaI/BamHI section of vector pET14b(Novagen, USA) to obtain vector pT14SS1SG.

Fig. 4 displays the above procedure for constructing vector pT14SS1SG.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pT14SS1SG to obtain a transformant designated *E. coli* HM 10310.

10

(Step 4) Construction of a vector containing a gene encoding STII/hG-CSF fusion protein

15 The first codon of the modified hG-CSF gene of plasmid pT14SS1SSG obtained in Step 3 was replaced by Thr in accordance with a site-directed mutagenesis(Papworth, C. et al., Strategies, 9, 3(1996)), which was conducted by PCR of the plasmid with a sense primer(SEQ ID NO: 12) having a modified nucleotide sequence, a complementary antisense primer(SEQ ID NO: 13), and pfu(Stragene, USA).

20 The amplified DNA fragment was recovered and restriction enzyme DpnI was added thereto to remove unconverted plasmids.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pT14SSG which contained a
25 gene having Thr in place of the first amino acid of hG-CSF(SEQ ID NO: 11).

-5 -4 -3 -2 -1 +1 +2 +3 +4 +5
Thr Asn Ala Tyr Ala Thr Pro Leu Gly Pro (SEQ ID NO: 11)
- ACA-AAT-GCC-TAC-GCG-ACA-CCC-CTG-GGC-CCT (SEQ ID NO: 12)
30 - TGT-TTA-CGG-ATG-CGC-TGT-GGG-GAC-CCG-GGA (SEQ ID NO: 13)

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pT14SSG to obtain a transformant designated *E. coli* HM 10301.

35

(Step 5) Construction of a vector containing a gene encoding modified STII /hG-CSF

5 Vector pT14SSG obtained in Step 4 was subjected to PCR using the complementary primers of SEQ ID NOS: 15 and 16, which were designed to substitute Thr codon for the 4th codon of STII in accordance with the procedure of Step 4 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid which contained a gene having Thr in place of the 4th amino acid of STII(SEQ ID NO: 14).

Met Lys Lys Thr Ile Ala Phe Leu (SEQ ID NO: 14)
5'-GG-TGT-TTT-ATG-AAA-AAG-ACA-ATC-GCA-TTT-CTT-C-3' (SEQ ID NO: 15)
15 3'-CC-ACA-AAA-TAC-TTT-TTC-TGT-TAG-CGT-AAA-GAA-G-5' (SEQ ID NO: 16)

The plasmid thus obtained was cleaved with XbaI and MluI, and then inserted at the XbaI/MluI section of vector pT14SSG obtained in step 4 to obtain vector pT14SSG-4T.

20

(Step 6) Construction of a vector containing a gene encoding modified STII /hG-CSF

25 Vector pT14SSG-4T obtained in Step 5 was subjected to PCR using the complementary primers of SEQ ID NOS: 18 and 19, which were designed to substitute Gln codon for the 22nd codon of STII in accordance with the procedure of Step 4 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pT14SSG-4T22Q which contained a gene having Gln in place of the 22nd amino acid of STII(SEQ ID NO: 17).

ASN Ala Gln Ala Thr Pro Leu Gly (SEQ ID NO: 17)
35 5'-CA-AAT-GCC-CAA-GCG-ACA-CCC-CTG-GGC-3' (SEQ ID NO: 18)
3'-GT-TTA-CGG-GTT-CGC-TGT-GGG-GAC-CCG-5' (SEQ ID NO: 19)

(Step 7) Construction of a vector containing a modified STII SD and a gene encoding modified STII /hG-CSF

5 Vector pT14SSG-4T22Q obtained in Step 6 was subjected to PCR using the complementary primers of SEQ ID NOS: 20 and 21 in accordance with the procedure of Step 4 to obtain vector pT140SSG-4T22Q having the six nucleotide sequences between the STII SD sequence(GAGG) and the initiation codon of STII(modified STII SD of SEQ ID NO: 71).

10 Fig. 5 represents the above procedure for constructing vector pT140SSG-4T22Q.

E. coli BL21(DE3) was transformed with vector pT140SSG-4T22Q to obtain a transformant designated *E. coli* HM 10302.

15 Example 3: Construction of a vector containing a gene encoding modified hG-CSF

To prepare a modified hG-CSF gene, S1 oligomer(SEQ ID NO: 22) having *E. coli*-preferred codons and Ser in place of the 17th amino acid of hG-CSF and AS1 oligomer(SEQ ID NO: 23) were synthesized using DNA synthesizer(Model 380B, Applied Biosystem, USA).

20 0.5 μ l(50 pmole) quantities of the oligonucleotides were reacted at 95 °C for 15 min. and kept until 35 °C for 3 hours. The mixture was precipitated in ethanol and subjected to gel electrophoresis(SDS-PAGE) to obtain a cohesive ended double strand(ds) oligomer.

25 The plasmid pT14SS1SG obtained in step 3 of Example 2 was cleaved with ApaI and BstXI, and then ligated with the adhesive-ended ds oligomer, to obtain vector pT14SS1S17SEG. Vector pT14SS1S17SEG contained a gene encoding hG-CSF having *E. coli*-preferred codons at the amino terminus and Ser in place of the 1st and 17th amino acids of hG-CSF, respectively.

30 Fig. 6 illustrates the above procedure for constructing vector pT140SS1S17SEG.

E. coli BL21(DE3) was transformed with vector pT14SS1S17SEG to obtain a transformant designated *E. coli* HM 10311, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24, 1999 under accession number KCCM-10154.

Example 4: Construction of vector containing a gene encoding *E. coli* OmpA signal peptide and modified hG-CSF

5 A vector containing a gene encoding Tac promoter and OmpA signal peptide(SEQ ID NO: 24) as well as a gene encoding modified hG-CSF was prepared as follows:

Met-Lys-Lys-Thr-Ala-Ile-Ala-Ile-Ala-Val-Ala-Leu-Ala-Gly-Phe-Ala-
 Thr-Val-Ala-Gln-Ala- (SEQ ID NO: 24)
 10 --GTT-GCG-CAA-GCT-TCT-CGA-- (SEQ ID NO: 25)
 --CAA-CGC-GTT-CGA-AGA-GCT-- (SEQ ID NO: 26)
 HindIII restriction site

15 Vector pT-CSF obtained in Example 1 was subjected to PCR using a primer(SEQ ID NO: 27) designed to substitute Ser codon for the 1st codon of hG-CSF and another primer(SEQ ID NO: 28), to provide an EcoRI restriction site(5'-GAATTC-3') downstream from the termination codon thereof to obtain a DNA fragment containing a gene encoding modified hG-CSF .

20 The DNA fragment was cleaved with HindIII and EcoRI, and then inserted at the HindIII/EcoRI section of vector pFlag.CTS(Eastman, USA) to obtain vector pTO1SG which contained a gene encoding *E. coli* OmpA signal peptide and modified hG-CSF(SEQ ID NO: 29).

Fig. 7 exhibits the above procedure for constructing vector pTO1SG.

25 *E. coli* BL21(DE3)(Stratagene, USA) was transformed with vector pTO1SG to obtain a transformant designated *E. coli* HM 10409.

Example 5: Construction of a vector containing a gene encoding *E. coli* OmpA signal peptide and modified hG-CSF

30 The first codon of the modified hG-CSF gene of plasmid pTO1SG obtained in Example 4 was replaced by Thr in accordance with site-directed mutagenesis(Papworth, C. et al., *Strategies*, 9, 3(1996)), by conducting PCR of the plasmid pTO1SG obtained in Example 4 with a sense primer(SEQ ID NO: 30) designed to substitute Thr codon for the 1st codon of hG-CSF and a
 35 complementary antisense primer(SEQ ID NO: 31).

E. coli XL-1 blue(Novagen, USA) was transformed with the modified

plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained plasmid pTOG which contained a gene having Thr in place of the first amino acid of hG-CSF.

5 *E. coli* BL21(DE3)(Stratagene, USA) was transformed with vector pTOG to obtain a transformant designated *E. coli* HM 10401.

Example 6: Production of modified hG-CSFs

(a) Production of [Ser1, Ser17] hG-CSF

10 Vector pTO1SG obtained in Example 4 was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

15 *E. coli* XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined and thus obtained was plasmid pTO1S17SG which contained a gene having Ser in place of the 1st and 17th amino acids of hG-CSF.

20 *E. coli* BL21(DE3)(Stratagene, USA) was transformed with vector pTO1S17SG to obtain a transformant designated *E. coli* HM 10410, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24, 1999 under accession number KCCM-10151.

(b) Production of [Ser17] hG-CSF

25 Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

30 *E. coli* XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pTO17SG which contained a gene having Ser in place of the 17th amino acid of hG-CSF.

35 *E. coli* BL21(DE3)(Stratagene, USA) was transformed with vector pTO17SG to obtain a transformant designated *E. coli* HM 10411, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24,

1999 under accession number KCCM-10152.

(c) Production of [Thr17] hG-CSF

5 Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 34) designed to substitute Thr codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 35) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

10 *E. coli* XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequences of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pTO17TG which contained a gene having Thr in place of the 17th amino acid of hG-CSF.

15 *E. coli* BL21(DE3)(Stratagene, USA) was transformed with vector pTO17TG to obtain a transformant designated *E. coli* HM 10413.

(d) Production of [Ala17] hG-CSF

20 Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 36) designed to substitute Ala codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 37) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

25 *E. coli* XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of DNA recovered from transformed colonies was determined, and thus obtained was plasmid pTO17AG which contained a gene having Ala in place of the 17th amino acid of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO17AG to obtain a transformant designated *E. coli* HM 10414.

(e) Production of [Gly17] hG-CSF

30 Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 38) designed to substitute Gly codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 39) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

35 *E. coli* XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies

was determined, and thus obtained was plasmid pTO17GG which contained a gene having Gly in place of the 17th amino acids of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO17GG to obtain a transformant designated *E. coli* HM 10415.

5

(f) Production of [Asp17] hG-CSF

Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 40) designed to substitute Asp codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 41) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pTO17APG which contained a gene having Asp in place of the 17th amino acids of hG-CSF.

15

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO17APG to obtain a transformant designated *E. coli* HM 10416.

Example 7: Construction of a vector containing a gene encoding *E. coli* Gene III signal peptide and modified hG-CSF

20

(a) Construction of a vector containing a gene encoding arabinose promoter and *E. coli* Gene III signal peptide

25

A vector containing a gene encoding arabinose promoter and *E. coli* Gene III signal peptide(SEQ ID NO: 42) as well as a gene encoding modified hG-CSG was prepared as follows:

Met-Lys-Lys-Leu-Leu-Phe-Ala-Ile-Pro-Leu-Val-Val-Pro-
 30 Phe-Tyr-Ser-His-Ser- (SEQ ID NO: 42)
 -TAT-AGC-CAT-AGC-ACC-ATG-GAG- (SEQ ID NO: 43)
 -ATA-TCG-GTA-TCG-TGG-TAC-CTC- (SEQ ID NO: 44)

NcoI restriction site

35

Plasmid pBAD · gIIIA(Invitrogen, USA) containing a gene encoding arabinose promoter and Gene III signal peptide was cleaved with NcoI, and

single stranded DNAs were removed with Klenow DNA polymerase to obtain a blunt-ended double stranded DNA, which was then cleaved with BglII to obtain a vector fragment having both blunt end and a cohesive end.

Vector pT-CSF obtained in Example 1 was subjected to PCR using a
 5 sense primer(SEQ ID NO: 46) having a nucleotide sequence coding for the 2nd
 to the 9th amino acids of hG-CSF(SEQ ID NO: 45) and a complementary
 antisense primer(SEQ ID NO: 47) in accordance with the procedure of Step 4 of
 Example 2 to obtain a blunt-ended DNA fragment containing hG-CSF gene and
 a BamHI restriction site in the carboxy terminus. The fragment then was
 10 cleaved with BamHI to obtain hG-CSF gene fragment having both a blunt end
 and a cohesive end.

	Pro Leu Gly Pro Ala Ser Ser Leu	(SEQ ID NO 45)
5'	-C-CCC-CTG-GGC-CCT-GCC-AGC-TCC-CTG-3'	(SEQ ID NO 46)
15	3' -G-GGG-GAC-CCG-GGA-CGG-TCG-AGG-GAC-5'	(SEQ ID NO 47)

The hG-CSF gene fragment as inserted into the vector obtained above
 to obtain vector pBADG which contained a gene encoding *E. coli* Gene III
 signal peptide and hG-CSF(SEQ ID NO: 48).

20 Fig. 8 describes the above procedure for constructing vector pBADG.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector
 pBADG to obtain a transformant designated *E. coli* HM 10501.

(b) Production of [Met2, Val3] hG-CSF

25

Plasmid pBAD · gIIIA(Invitrogen, USA) was cleaved with NcoI and
 BglII to obtain a fragment having two cohesive ends.

Vector pT-CSF obtained in Example 1 was subjected to PCR using a
 sense primer(SEQ ID NO: 50) having a nucleotide sequence coding for the 1st
 30 to the 9th amino acids of [Met2, Val3] hG-CSF(SEQ ID NO: 49) and a
 complementary antisense primer(SEQ ID NO: 51) in accordance with the
 procedure of Step 4 of Example 2 to obtain a blunt-ended DNA fragment
 containing hG-CSF gene and a BamHI restriction site in the carboxy terminus,
 which was then was cleaved with Neol and BamHI to obtain a hG-CSF gene
 35 fragment having two cohesive ends.

Thr Met Val Gly Pro Ala Ser Ser Leu (SEQ ID NO: 49)
5'-TAC-GCG-TCC-ATG-GTG-GGC-CCT-GCC-AGC-TCC-CTG-3' (SEQ ID NO: 50)
3'-ATG-CGC-AGG-TAC-CAC-CCG-GGA-CGG-TCG-AGG-GAC-5' (SEQ ID NO: 51)

5 NcoI restriction site

The hG-CSF gene fragment was inserted into the vector obtained above to obtain vector pBAD2M2VG contained a gene coding *E. coli* Gene III signal peptide, and Met and Val in place of the 2nd and 3rd amino acids of hG-CSF (SEQ ID NO: 52), respectively.

Fig. 9 shows the above procedure for constructing vector pBAD2M3VG.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pBAD2M3VG to obtain a transformant designated *E. coli* HM 10510, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24, 1999 under accession number of KCCM-10153.

(c) Production of [Ser17] hG-CSF

20 Vector pBADG obtained in (a) was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

25 *E. coli* XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pBAD17SG which contained a gene having Ser in place of the 17th amino acid of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pBAD17SG to obtain a transformant designated *E. coli* HM 10511.

30

(d) Production of [Met2, Val3, Ser17] hG-CSF

35 Vector pBAD2M3VG obtained in (b) was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified

plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pBAD2M3V17SG which
5 contained a gene having Met, Val and Ser in place of the 2nd, 3rd and 17th amino acids of hG-CSF, respectively.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pBAD2M3V17SG to obtain a transformant designated *E. coli* HM 10512.

10 Example 8: Production of hG-CSF

Transformants prepared in Examples 2 to 7 were cultured in LB medium(1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl) and then incubated in the presence of an expression inducer(IPTG) for 3 hours or
15 cultured in the absence of IPTG more than 15 hours. Each of the cultures was centrifuged at 6,000 rpm for 20 min. to precipitate bacterial cells, and the precipitate was suspended in a 1/10 volume of isotonic solution(20 % sucrose, 10 mM Tris-Cl buffer solution containing 1 mM EDTA, pH 7.0). The suspension was allowed to stand at room temperature for 30 min, and then
20 centrifuged at 7,000 rpm for 10 min. to collect bacterial cells. The cells were resuspended in D.W. at 4 °C and centrifuged at 7,000 rpm for 10 min. to obtain a supernatant as a periplasmic solution. The hG-CSF level in the periplasmic solution was assayed in accordance with ELISA method(Kato, K. et al., J. Immunol., 116, 1554(1976)) using an antibody against hG-CSF(Aland, USA),
25 which was calculated as the amount of hG-CSF produced per 1 ℓ of culture. The results are shown in Table I.

Table 1

Transformant	Example	Expression Vector	hG-CSF Level in periplasm(mg/ℓ)
HM 10301	2(Step 4)	pT14SSG	65
HM 10302	2(Step 7)	pT140SSG-4T22Q	277
HM 10310	2(Step 3)	pT14SS1SG	92
HM 10311	3	pT14SS1S17SEG	1,512
HM 10401	5	pTOG	85
HM 10409	4	pTO1SG	105
HM 10410	6(a)	pTO1S17SG	1,477
HM 10411	6(b)	pTO17SG	1,550
HM 10413	6(c)	pTO17TG	1,373
HM 10414	6(d)	pTO17AG	1,486
HM 10415	6(e)	pTO17GG	1,480
HM 10416	6(f)	pTO17APG	67
HM 10501	7(a)	pBADG	54
HM 10510	7(b)	pBAD2M3VG	69
HM 10511	7(c)	pBAD17SG	937
HM 10512	7(d)	pBAD2M3V17SG	983

Example 9: Purification of hG-CSF

5

Transformant *E. coli* HM 10411 prepared in Example 6(b) was cultured in LB medium and the culture was centrifuged for 6,000 rpm for 20 min. to harvest cells. The periplasmic solution was prepared from the cells by repeating the procedure of Example 8.

10

The periplasmic solution was adjusted to pH 5.0 to 5.5, adsorbed on a CM-Sepharose(Pharmacia Inc., Sweden) column pre-equilibrated to pH 5.3, and then, the column was washed with 25 mM NaCl. hG-CSF was eluted by sequentially adding to the column buffer solutions containing 50mM, 100mM and 200mM NaCl, and fractions containing hG-SCF were collected and

15

combined. The combined fractions were subjected to Phenyl Sepharose(Pharmacia Inc., Sweden) column chromatography to obtain [Ser17] hG-CSF having a purity of 99%.

Further, the above procedure was repeated using each of the transformants *E. coli* HM 10311, HM 10409, HM 10411, HM 10413, HM 10414, HM 10415, HM 10510 and HM 10512 prepared in Examples 3, 4, 6(b), 6(c), 6(d), 6(e), 7(b) and 7(d), respectively.

5 Each of the purified hG-CSF fraction was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis(SDS-PAGE) to determine the purity and approximate concentration of the hG-CSF, and then subjected to ELISA to determine the exact hG-CSF concentration in the periplasmic solution. Met-hG-CSF(Kirin amgen) was used as a control.

10 Fig. 10a reproduces the SDS-PAGE result, wherein lane 1 shows Met-G-CSF, lane 2, the periplasmic solution of the transformant *E. coli* HM 10411, and lane 3, the purified [Ser17] hG-CSF. As can be seen from Fig. 10b, the molecular weight of [Ser17] hG-CSF is the same as that of wild-type hG-CSF and the periplasmic solution of the transformant *E. coli* HM 10411 contains a
15 high level of [Ser17] hG-CSF.

Further, the N-terminal amino acid sequences of hG-CSFs were determined and the nucleotide sequences coding for the 1st to 32nd amino acids produced using the transformants HM 10311, HM 10409, HM 10411, HM 10413, HM 10414, HM 10415, HM 10510 and HM 10512 shown in SEQ ID
20 NOS: 56, 58, 60, 62, 64, 66, 68 and 70, respectively. The result shows that the modified hG-CSF produced according to the present invention is not methionylated at N-terminus.

A nitrocellulose filter (Bio-Rad Lab., USA) was wetted with a buffer solution for blotting(170 mM glycine, 25mM Tris · HCl(pH 8), 20% methanol)
25 and the proteins separated on the gel were western blotted onto a nitrocellulose filter(Bio-Rad Lab., USA.) for 3 hours. The filter was kept in 1% Casein for 1 hour and was washed three times with PBS containing 0.05% Tween 20. The filter was put in a goat anti-G-CSF antibody(R&D System, AB-214-NA, USA) solution diluted with PBS and reacted at room temperature for 2 hours. After
30 reaction, the filter was washed 3 times with a PBST solution to remove unreacted antibody. Horseradish peroxidase-conjugated rabbit anti-goat IgG(Bio-Rad Lab., USA) diluted with PBS was added thereto and reacted at room temperature for 2 hour. The filter was washed with PBST, and a peroxidase substance kit(Bio-Rad Lab., USA) solution was added thereto to
35 develop a color reaction. The results from the above western blotting are shown in Fig. 10b, wherein lane 1 represents a positive control, Met-G-CSF,

and lane 2, purified [Ser17] hG-CSF. As can be seen from Fig. 10b, the molecular weight of [Ser17] hG-CSF equals that of wild-type hG-CSF.

Example 10: Cellular Activity of hG-CSF and Modified hG-CSF

5

Cell line HL-60(ATCC CCL-240 derived from the bone marrow of a promyelocytic leukemia patient/a white 36-year-old woman) was cultured in RPMI 1640 media containing 10% fetal bovine serum and adjusted to 2.2×10^5 cells/ml, followed by adding thereto DMSO(dimethylsulfoxide, culture grade/SIGMA) to a concentration of 1.25%(v/v). 90 μl of the resulting solution was added to a 96 well plate(Corning/low evaporation 96 well plate) in an amount of 2×10^4 cells/well and incubated at 37°C under 5% CO₂ for 48 hours.

Each of the modified [Ala17] hG-CSF, [Gly 17] hG-CSF, [Ser17] hG-CSF, and [Thr 17] hG-CSF was diluted in RPMI 1640 media to a concentration of 500 ng/ml and then serially diluted 10 times by 2-fold with RPMI 1640 media.

The resulting solution was added to wells at 10 μl per well and incubated at 37 °C for 48 hours. As a positive control, a commercially available hG-CSF(Jeil Pharmaceutical.).

The level of cell line increased was determined using a commercially available CellTiter96TM(Cat # G4100, Promega) based on the measured optical density at 670 nm.

As can be seen from Fig. 11, the cellular activities of the modified hG-CSFs are the same as, or higher than of that the positive control, wild-type hG-CSF.

While the embodiments of the subject invention have been described and illustrated, it is obvious that various changes and modifications can be made therein without departing from the spirit of the present invention which should be limited only by the scope of the appended claims.

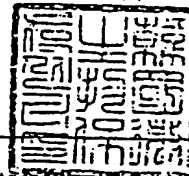
BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd
#893-5 Hajeo-ri Paltan-myun
Hwasung-Kun
Kyonggi-do,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
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I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>HM10311</i>	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10154
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March. 24. 1999 (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodazemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: April. 6. 1999



¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

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Hwasung-Kun
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#893-5 Hajeo-ri Paltan-myun
Hwasung-Kun
Kyonggi-do,
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What is claimed is :

1. A modified human granulocyte-colony stimulating factor(hG-CSF) which is characterized in that at least one of the 1st, 2nd, 3rd and 17th amino acids of wild-type hG-CSF(SEQ ID NO: 2) is replaced by other amino acid(s).
2. The modified hG-CSF of claim 1 whose amino acid sequence is the same as that of wild-type hG-CSF, except that
 - (a) the 1st amino acid is Ser;
 - (b) the 1st amino acid is Ser and the 17th amino acid is X;
 - (c) the 2nd amino acid is Met and the 3rd amino acid is Val;
 - (d) the 2nd amino acid is Met, the 3rd amino acid is Val and the 17th amino acid is X; or
 - (f) the 17th amino acid is X,wherein X is an amino acid which is not charged at neutral pH.
3. The modified hG-CSF of claim 2, wherein X is Ser, Thr, Ala or Gly.
4. The modified hG-CSF of claim 3, wherein X is Ser.
5. A DNA encoding the modified hG-CSF of any one of claims 1 to 4.
6. The DNA of claim 5, wherein the 1st to the 60th nucleotide sequence of the modified hG-CSF DNA corresponds to one selected from the group consisting of SEQ ID NOS: 55, 57, 59, 61, 63, 65, 67 and 69.
7. An expression vector comprising the DNA of claim 5.
8. The expression vector of claim 7, which further comprises a polynucleotide encoding a signal peptide attached at the 5'-end of the DNA encoding the modified hG-CSF.
9. The expression vector of claim 8, wherein the signal peptide is

E. coli thermoresistant enterotoxin II signal peptide or modified *E. coli* thermoresistant enterotoxin II signal peptide.

10. The expression vector of claim 9, wherein the *E. coli*
5 thermoresistant enterotoxin II signal peptide has the amino acid sequence of SEQ ID NO: 53.

11. The expression vector of claim 9, wherein the modified *E. coli*
10 thermoresistant enterotoxin II signal peptide has the amino acid sequence of SEQ ID NO: 54.

12. The expression vector of claim 9, which further comprises a
15 modified *E. coli* enterotoxin II Shine-Dalgarno sequence having the nucleotide sequence of SEQ ID NO: 71.

13. The expression vector of claim 8, wherein the signal peptide is
E. coli beta lactamase signal peptide or modified *E. coli* beta lactamase signal
peptide.

14. The expression vector of claim 13, wherein the *E. coli* beta
20 lactamase signal peptide has the amino acid sequence of SEQ ID NO: 24.

15. The expression vector of claim 8, wherein the signal peptide is
E. coli Gene III signal peptide or modified *E. coli* Gene III signal peptide.
25

16. The expression vector of claim 15, wherein the *E. coli* Gene III
signal peptide has the amino acid sequence of SEQ ID NO: 42.

17. The expression vector of claim 7 or 8, which is pT14SS1SG,
30 pT14SS1S17SEG, pTO1SG, pTO1S17SG, pTO17SG or pBAD2M3V17SG.

18. A microorganism transformed with the expression vector
according to claim 7 or 8.

19. The microorganism of claim 18, which is a transformed *E. coli*.
35

20. The microorganism of claim 19, wherein the transformed *E. coli* is *E. coli* BL21(DE3)/pT14SS1SG(HM 10310), *E. coli* BL21(DE3)/pT14SS1S17SEG(HM 10311, KCCM-10154), *E. coli* BL21(DE3)/pTO1SG(HM 10409), *E. coli* BL21(DE3)/pTO1S17SG(HM 10410, 5 KCCM-10151), *E. coli* BL21(DE3)/pTO17SG(HM 10411, KCCM-10152), *E. coli* BL21(DE3)/pTO17TG(HM 10413), *E. coli* BL21(DE3)/pTO17AG(HM 10414), *E. coli* BL21(DE3)/pTO17GG(HM 10415), *E. coli* BL21(DE3)/pBAD2M3VG(HM 10510, KCCM-10153), *E. coli* BL21(DE3)/pBAD17SG(HM 10511) or *E. coli* 10 BL21(DE3)/pBAD2M3V17SG(HM 10512).

21. A process for producing a modified hG-CSF in microorganism which comprises culturing the transformed microorganism of claim 18 to produce and secrete the modified hG-CSF to periplasm. 15

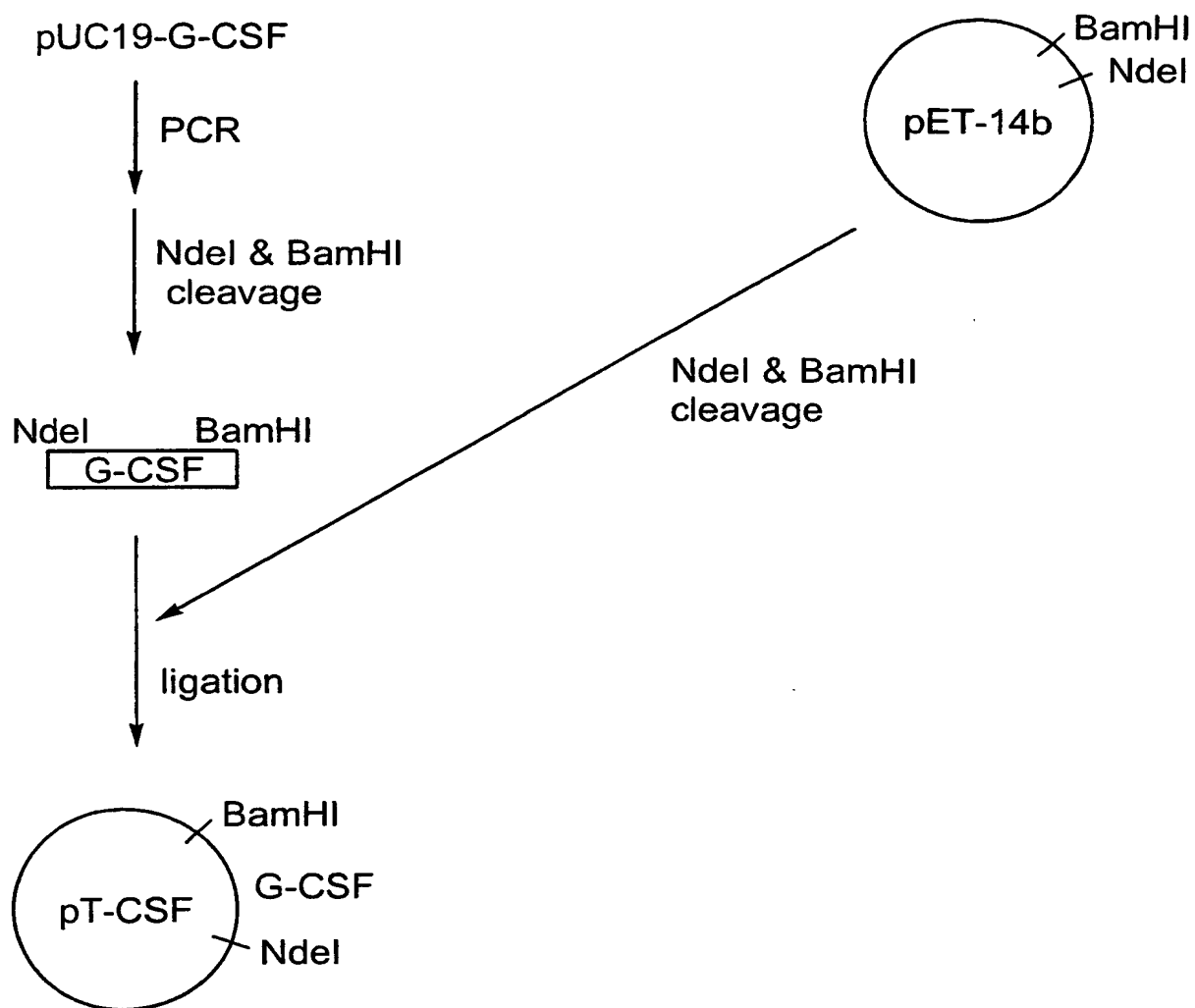
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Fig. 1

T	P	L	G	P	A	S	S	L	P	Q	S	F	L	L	K
aca	ccc	ctg	ggc	cct	gcc	agc	tcc	ctg	ccc	cag	agc	ttc	ctg	ctc	aag
C	L	E	Q	V	R	K	I	Q	G	D	G	A	A	L	Q
tgc	tta	gag	caa	gtg	agg	aag	atc	cag	ggc	gat	ggc	gca	gcg	ctc	cag
E	K	L	C	A	T	Y	K	L	C	H	P	E	E	L	V
gag	aag	ctg	tgt	gcc	acc	tac	aag	ctg	tgc	cac	ccc	gag	gag	ctg	gtg
L	L	G	H	S	L	G	I	P	W	A	P	L	S	S	C
ctg	ctc	gga	cac	tct	ctg	ggc	atc	ccc	tgg	gct	ccc	ctg	agc	tcc	tgc
P	S	Q	A	L	Q	L	A	G	C	L	S	Q	L	H	S
ccc	agc	cag	gcc	ctg	cag	ctg	gca	ggc	tgc	tig	agc	caa	ctc	cat	agc
G	L	F	L	Y	Q	G	L	L	Q	A	L	E	G	I	S
ggc	ctt	ttc	ctc	tac	cag	ggg	ctc	ctg	cag	gcc	ctg	gaa	ggg	ata	tcc
P	E	L	G	P	T	L	D	T	L	Q	L	D	V	A	D
ccc	gag	tig	ggt	ccc	acc	tig	gac	aca	ctg	cag	ctg	gac	gtc	gcc	gac
F	A	T	T	I	W	Q	Q	M	E	E	L	G	M	A	P
ttt	gcc	acc	acc	atc	tgg	cag	cag	atg	gaa	gaa	ctg	gga	atg	gcc	cct
A	L	Q	P	T	Q	G	A	M	P	A	F	A	S	A	F
gcc	ctg	cag	ccc	acc	cag	ggt	gcc	atg	ccg	gcc	ttc	gcc	tct	gct	ttc
Q	R	R	A	G	G	V	L	V	A	S	H	L	Q	S	F
cag	cgc	cgg	gca	gga	ggg	gtc	ctg	gtt	gct	agc	cat	ctg	cag	agc	ttc
L	E	V	S	Y	R	V	L	R	H	L	A	Q	P		
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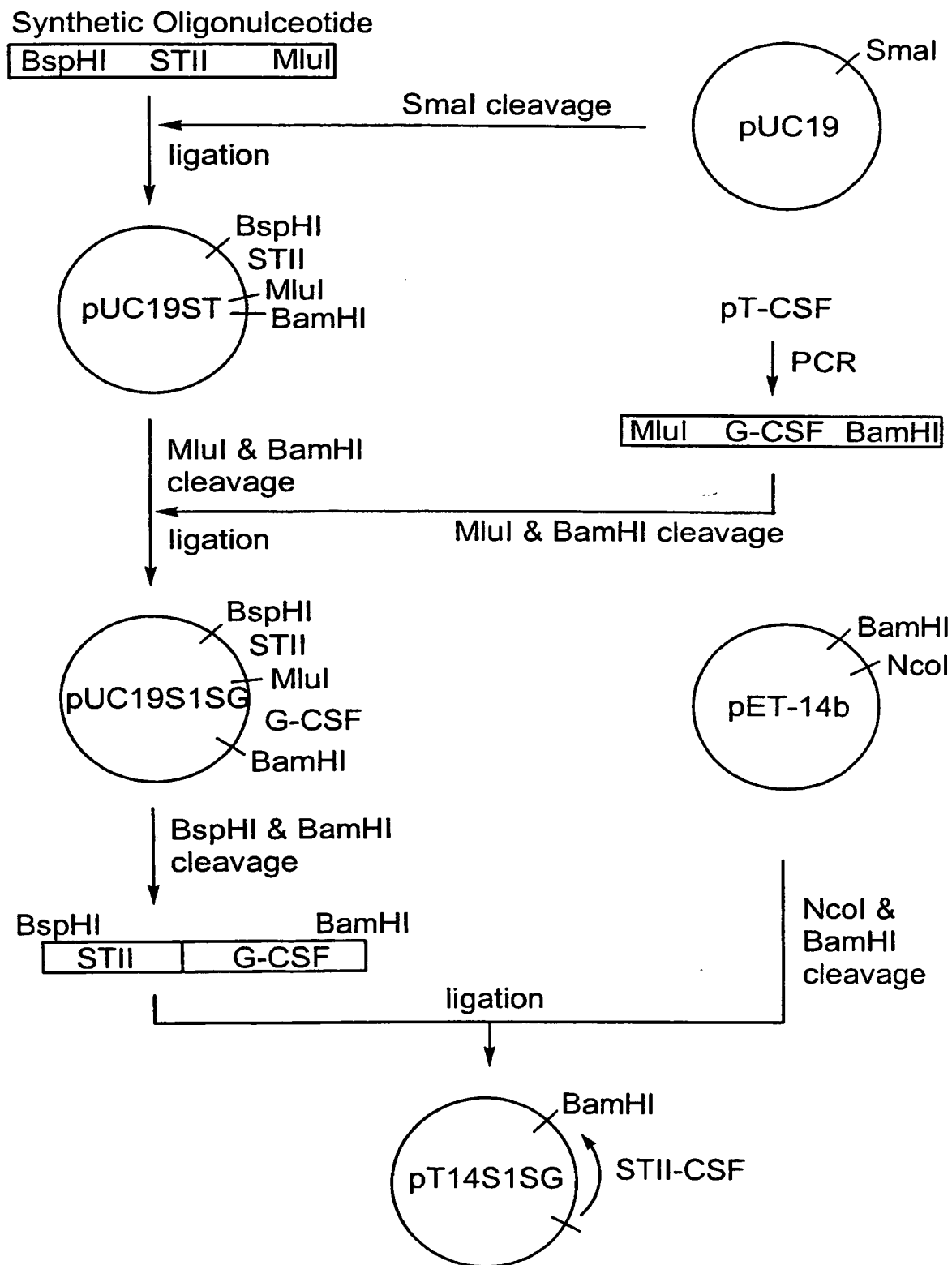
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Fig. 2



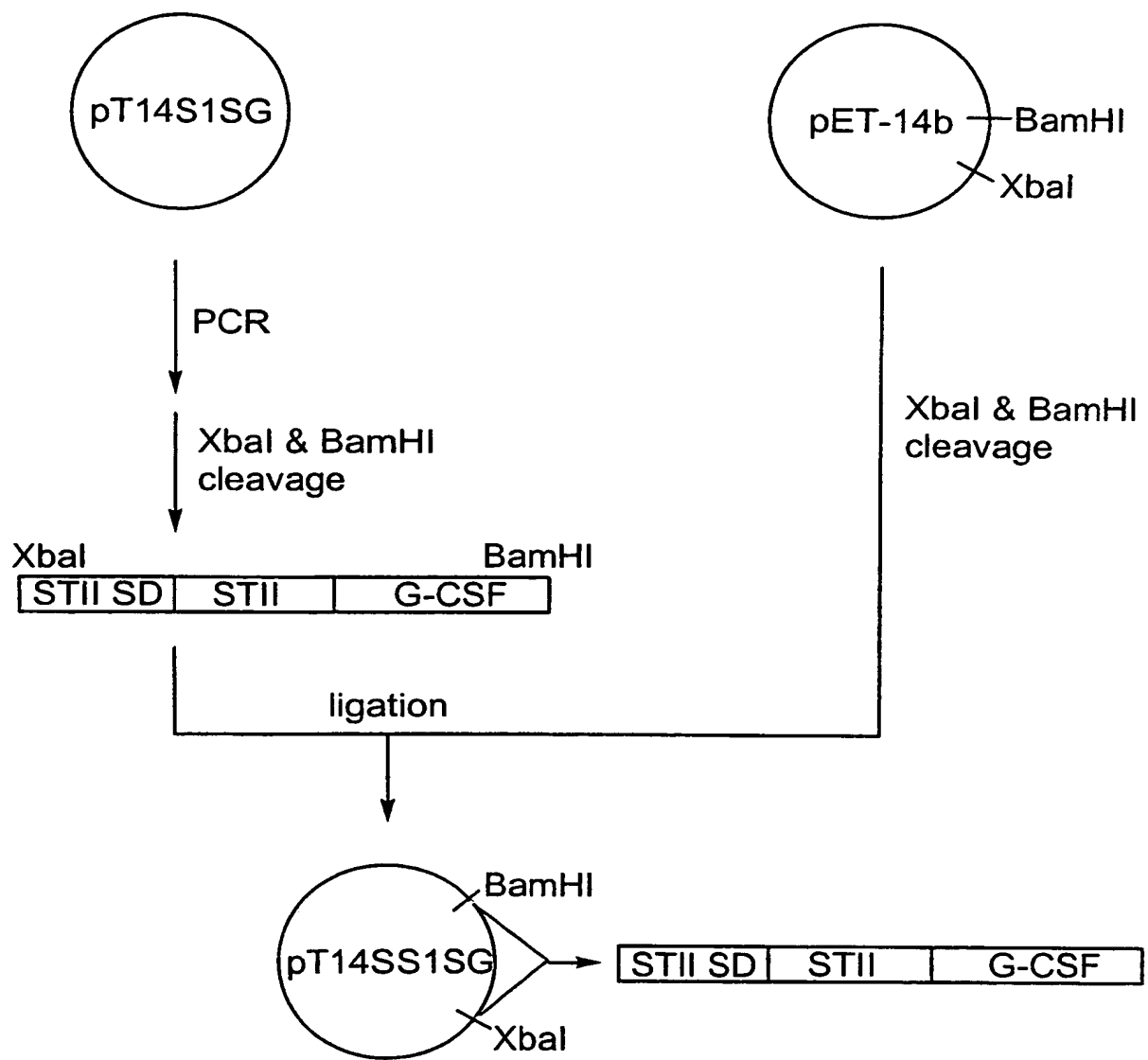
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Fig. 3



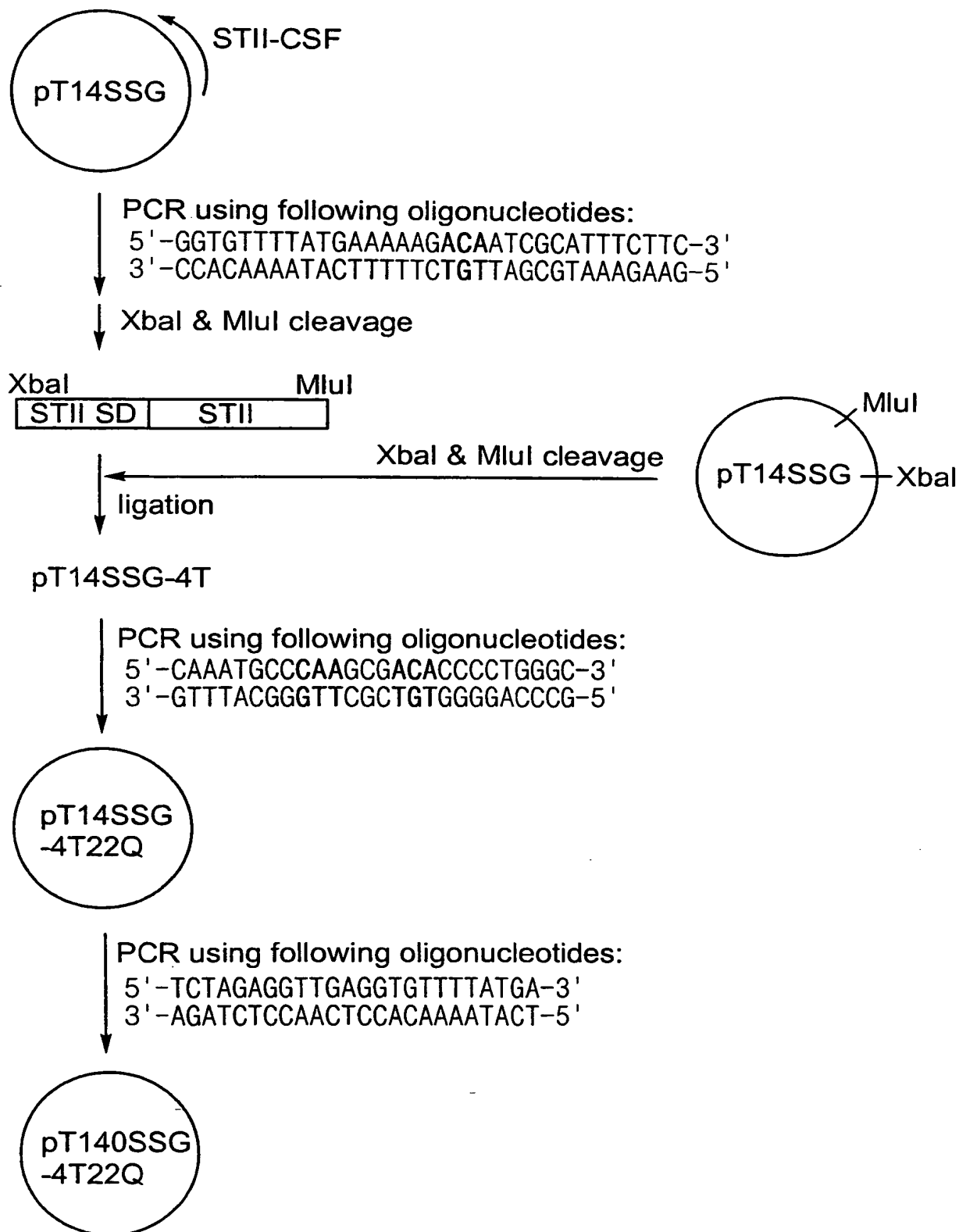
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Fig. 4



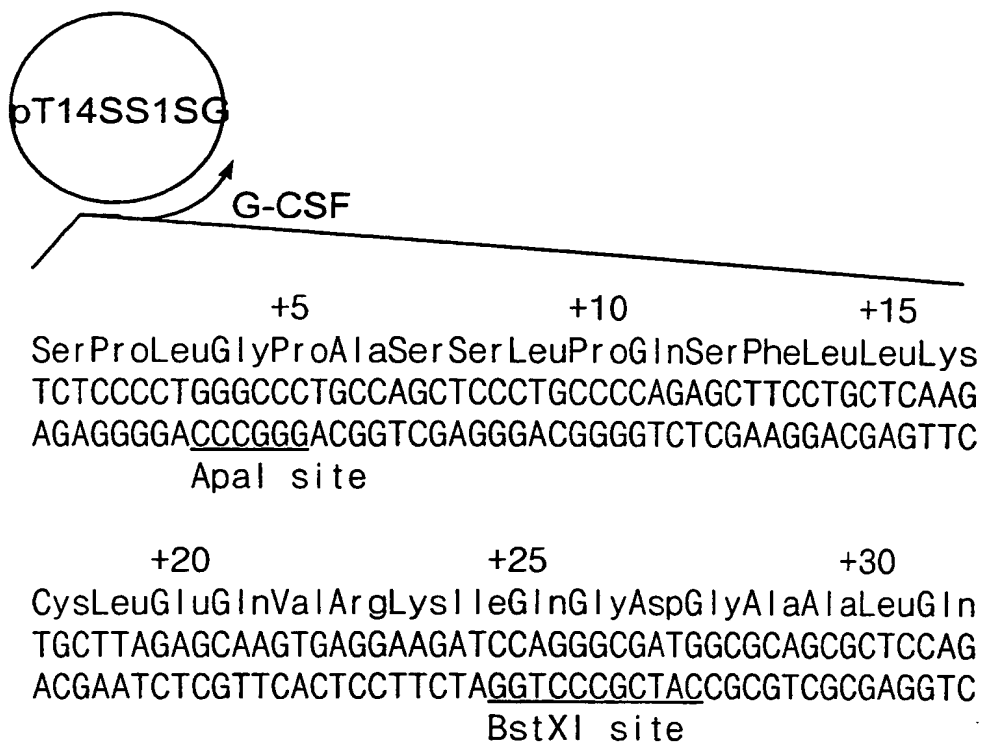
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Fig. 5



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Fig. 6



ApaI & BstXI cleavage
 followed by recovery of vector fragment

← annealing oligonucleotides

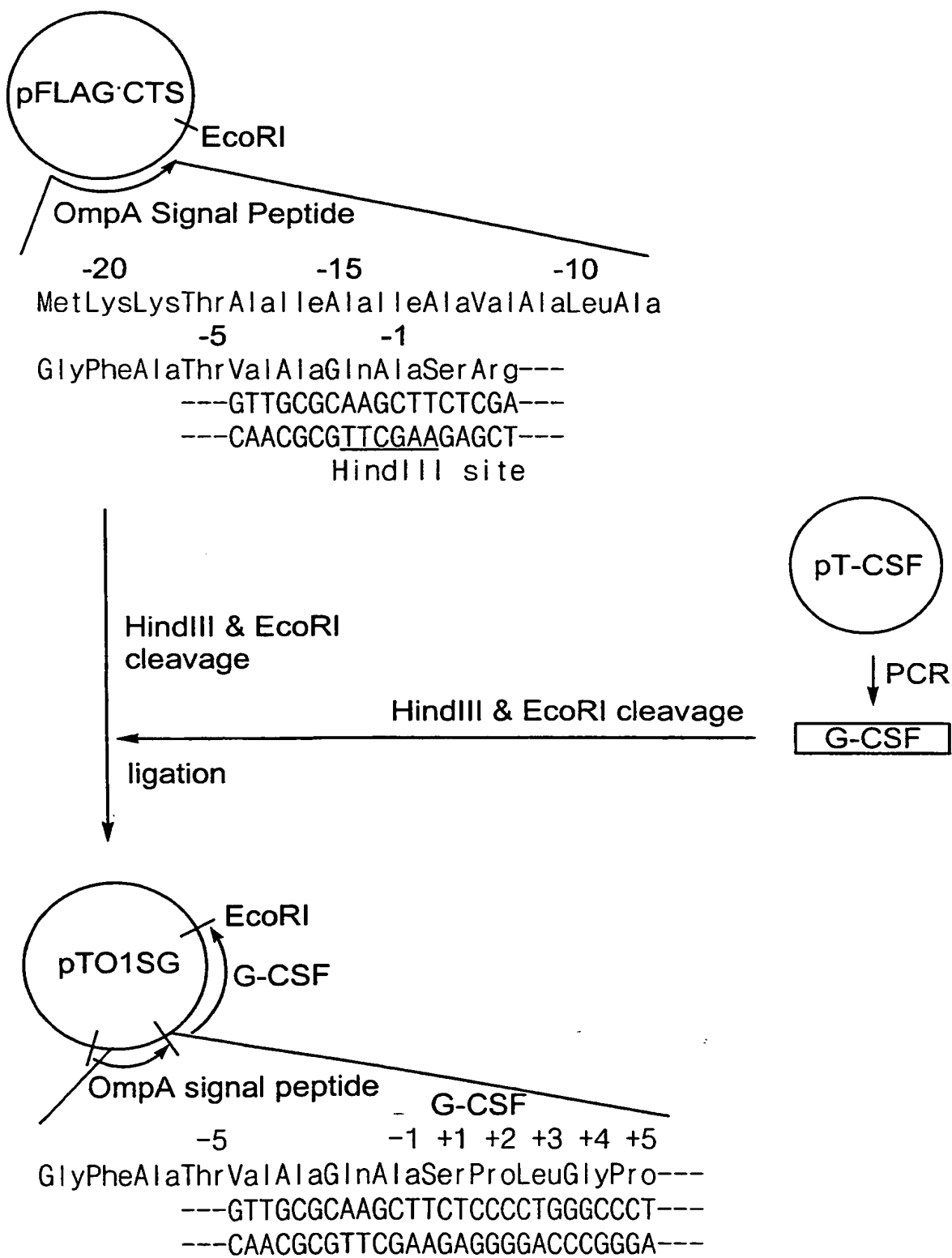
5' -CAGCCTCTTCTCTTCCACAATCTTTCTTCTTAAGTCTCTTGAACAA
 3' -CCGGGTCGGAGAAGAGAAGGTGTTAGAAAGGAAGAATTCAGAGAACTTGTT
 GTTAGAAAGATCCAAGGCG-3'
 CAATCTTTCTAGGTT-5'

ligation

pT14SS1S17SEG

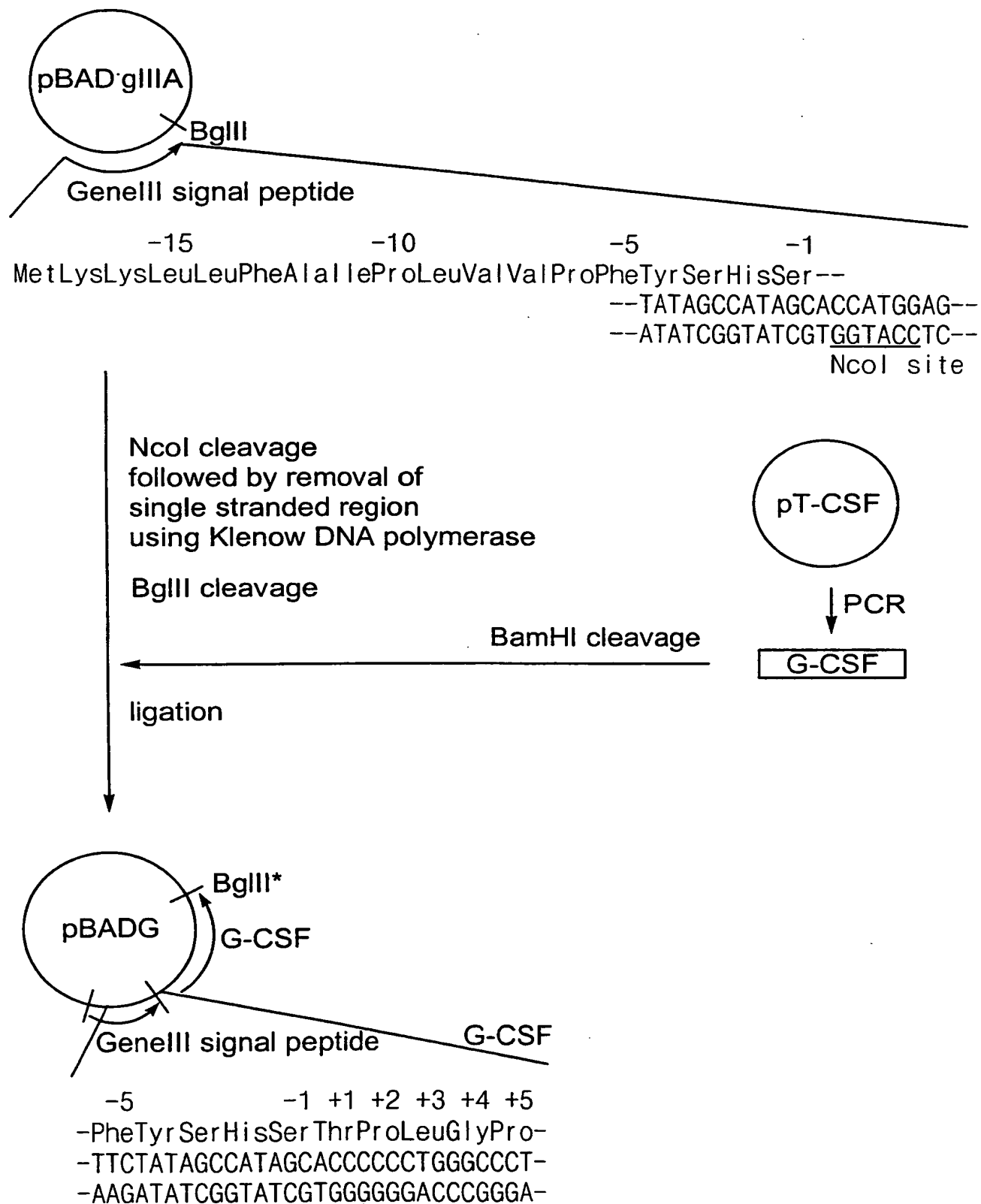
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Fig. 7



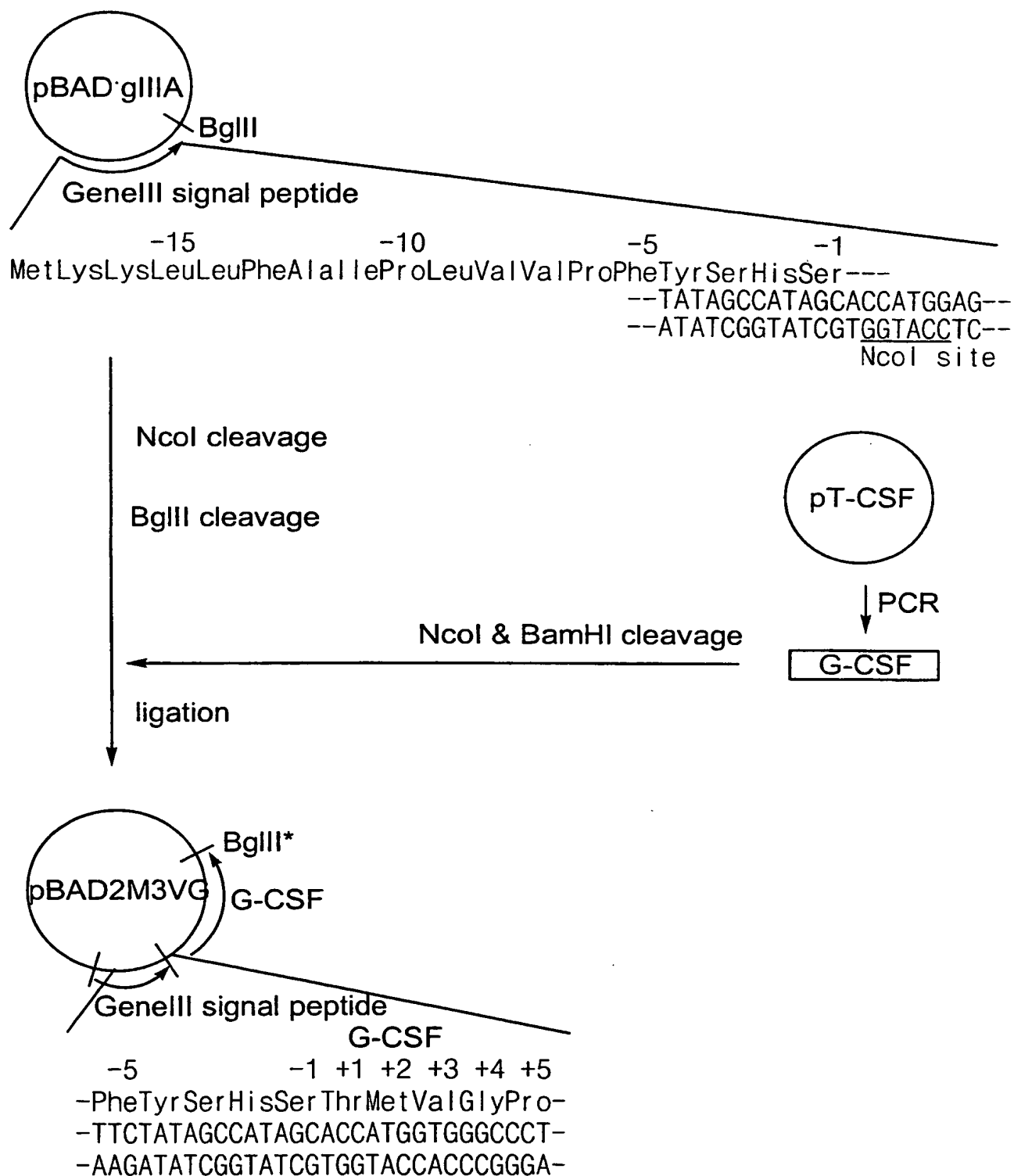
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Fig. 8



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Fig. 9



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Fig. 10A

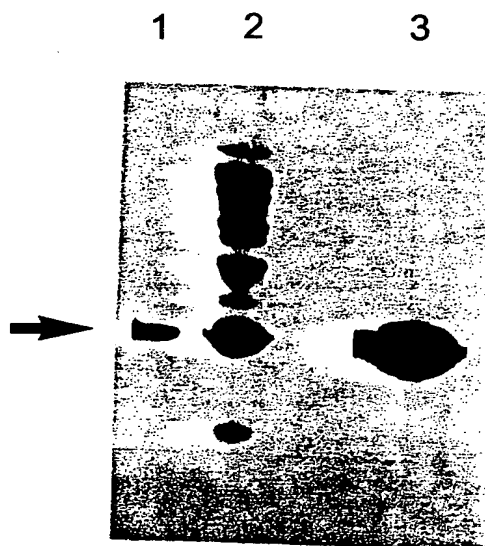
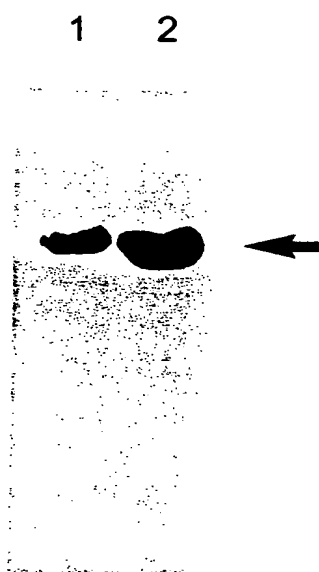
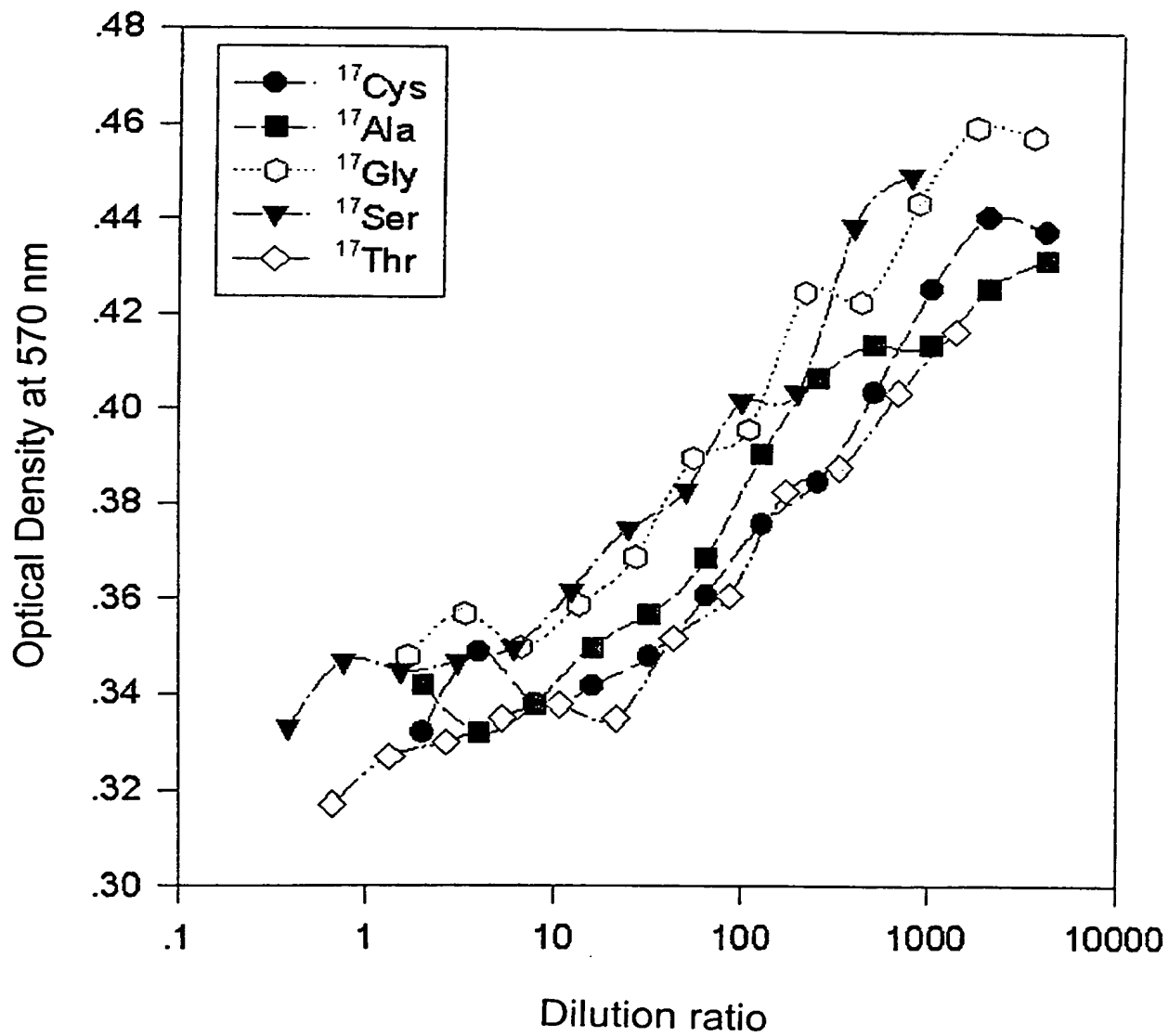


Fig. 10B



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Fig. 11



Sequence Listing

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 <170> KOPATIN 1.0
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 <211> 522
 <212> DNA
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 <221> CDS
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 tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
 Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30
 gag aag ctg tgt gcc acc tac aag ctg tgc cac ccc gag gag ctg gtg 144
 Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
 35 40 45
 ctg ctc gga cac tct ctg ggc atc ccc tgg gct ccc ctg agc tcc tgc 192
 Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
 50 55 60
 ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa ctc cat agc 240

2

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
 65 70 75 80

ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa ggg ata tcc 288
 Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
 85 90 95

ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac gtc gcc gac 336
 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp
 100 105 110

ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga atg gcc cct 384
 Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
 115 120 125

gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc tct gct ttc 432
 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
 130 135 140

cag cgc cgg gca gga ggg gtc ctg gtt gct agc cat ctg cag agc ttc 480
 Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
 145 150 155 160

ctg gag gtg tcg tac cgc gtt cta cgc cac ctt gcg cag ccc 522
 Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
 165 170

<210> 2

<211> 174

<212> PRT

<213> *Homo sapiens*

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 1 5 10 15

Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

3

Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
 35 40 45

Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
 50 55 60

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
 65 70 75 80

Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
 85 90 95

Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp
 100 105 110

Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
 115 120 125

Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
 130 135 140

Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
 145 150 155 160

Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
 165 170

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<211> 32

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<213> Artificial Sequence

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<211> 36
<212> DNA
<213> Artificial Sequence

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<223> Oligonucleotide primer for the C-terminal of hG-CSF

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<210> 5
<211> 72
<212> DNA
<213> Artificial Sequence

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<223> Oligonucleotide for preparing *E. coli* enterotoxin II signal peptide

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atgcctacgc gt 72

<210> 6
<211> 72
<212> DNA
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<210> 7

<211> 39

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<213> Artificial Sequence

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<223> Oligonucleotide primer coding for the N-terminal of [Ser1]hG-CSF

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<210> 8

<211> 42

<212> DNA

<213> Artificial Sequence

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<223> Oligonucleotide primer coding for the C-terminal of [Ser1]hG-CSF

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<210> 9

<211> 65

<212> DNA

<213> Artificial Sequence

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ctatg 65

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 <211> 45
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 <213> Artificial Sequence

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 <212> PRT
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 <223> Last five amino acids of *E. coli* enterotoxin II signal peptide plus the 1st to the 5th amino acids of hG-CSF

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 1 5 10

<210> 12
 <211> 30
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 <213> Artificial Sequence

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 <223> Oligonucleotide for preparing [Thr1]hG-CSF

<400> 12
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<210> 13
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 <212> DNA
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30

<210> 14
 <211> 8
 <212> PRT
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<220>
 <223> N-terminal sequence of *E. coli* enterotoxin II signal peptide
 having threonine as the 4th amino acid

<400> 14
 Met Lys Lys Thr Ile Ala Phe Leu
 1 5

<210> 15
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 <212> DNA
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 enterotoxin II signal peptide with threonine

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 ggtgttttat gaaaaagaca atcgcat ttc

33

8

<210> 16
 <211> 33
 <212> DNA
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 gaagaaatgc gattgtcttt ttcataaaac acc 33

<210> 17
 <211> 8
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 <223> C-terminal sequence of *E. coli* enterotoxin II signal peptide
 having glutamine as the 22nd amino acid

<400> 17
 Asn Ala Gln Ala Thr Pro Leu Gly
 1 5

<210> 18
 <211> 26
 <212> DNA
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<210> 19

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<211> 24
<212> DNA
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<223> Oligonucleotide for modifying *E. coli* enterotoxin II Shine-Dalgarno sequence
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tcataaaaca cctcaacctc taga 24

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10

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<223> S1 oligomer having *E. coli*-preferred nucleotide sequence coding for the 6th to 26th amino acids of [Ser17]hG-CSF

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aaggcg

66

<210> 23

<211> 66

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense of SEQ ID NO: 22 (AS1 oligomer)

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66

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<212> PRT

<213> *Escherichia coli*

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<222> (1).. (21)

<223> *E. coli* OmpA signal peptide

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1

5

-

10

15

Thr Val Ala Gln Ala

20

<210> 25
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<212> DNA
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<400> 25
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<211> 39
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<213> Artificial Sequence

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<400> 27
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<212> DNA

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accgaattct cagggtgctgcg caaggtggcg tagaacgcg

39

<210> 29

<211> 13

<212> PRT

<213> Artificial Sequence

<220>

<223> *E. coli* OmpA signal peptide plus the 1st to the 5th amino acids of [Ser1]hG-CSF

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1

5

10

<210> 30

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

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30

<210> 31

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<213> Artificial Sequence

13

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30

<210> 32

<211> 33

<212> DNA

<213> Artificial Sequence

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<223> Oligonucleotide for preparing [Ser17]hG-CSF

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33

<210> 33

<211> 33

<212> DNA

<213> Artificial Sequence

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<223> Antisense of SEQ ID NO: 32

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cctcacttgc tctaaagact tgagcaggaa gct

33

<210> 34

<211> 33

<212> DNA

<213> Artificial Sequence

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14

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<210> 39
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<210> 40
<211> 33
<212> DNA
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<220>
<223> Oligonucleotide for preparing [Asp17]hG-CSF

<400> 40
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<210> 41
<211> 33
<212> DNA
<213> Artificial Sequence

16

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<400> 41

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33

<210> 42

<211> 18

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<213> *Escherichia coli*

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<222> (1)..(18)

<223> *E. coli* Gene III signal peptide

<400> 42

Met	Lys	Lys	Leu	Leu	Phe	Ala	Ile	Pro	Leu	Val	Val	Pro	Phe	Tyr	Ser
1				5					10					15	

His Ser

<210> 43

<211> 21

<212> DNA

<213> Artificial Sequence

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<223> Oligonucleotide containing Nco I restriction site

<400> 43

tatagccata gcaccatgga g

21

<210> 44

<211> 21

<212> DNA

17

<213> Artificial Sequence

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<223> Antisense of SEQ ID NO: 43

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21

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<212> PRT

<213> Artificial Sequence

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<223> The 2nd to the 10th amino acids of hG-CSF

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Pro Leu Gly Pro Ala Ser Ser Leu

1

5

<210> 46

<211> 25

<212> DNA

<213> Artificial Sequence

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<223> Oligonucleotide primer coding for the 2nd to the 10th amino acids of hG-CSF plus an additional cytosine at its 5'-end

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25

<210> 47

<211> 25

<212> DNA

<213> Artificial Sequence

18

<220>

<223> Antisense of SEQ ID NO: 46

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25

<210> 48

<211> 10

<212> PRT

<213> Artificial Sequence

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<223> *E. coli* Gene III signal peptide plus the 1st to the 5th amino acids of hG-CSF

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Phe	Tyr	Ser	His	Ser	Thr	Pro	Leu	Gly	Pro
1				5				10	

<210> 49

<211> 9

<212> PRT

<213> Artificial Sequence

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<223> The 1st to the 9th amino acids of [Met2,Val3]hG-CSF

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1				5				

<210> 50

<211> 33

<212> DNA

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<220>

19

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33

<210> 51

<211> 33

<212> DNA

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<223> Antisense of SEQ ID NO: 50

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33

<210> 52

<211> 10

<212> PRT

<213> Artificial Sequence

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<223> *E. coli* Gene III signal peptide plus the 1st to the 5th amino acids of [Met2,Val3]hG-CSF

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1				5				10	

<210> 53

<211> 23

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<213> *Escherichia coli*

<220>

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<222> (1)..(23)

20

<223> Thermoresistant *E. coli* enterotoxin II signal peptide

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Ile Ala Thr Asn Ala Tyr Ala
20

<210> 54

<211> 23

<212> PRT

<213> Artificial Sequence

<220>

<223> Modified thermoresistant *E. coli* enterotoxin II signal peptide

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Ile Ala Thr Asn Ala Gln Ala
20

<210> 55

<211> 96

<212> DNA

<213> Artificial Sequence

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<223> Nucleotide sequence coding for the 1st to 32nd amino acids of [Ser1, Ser17]hG-CSF

<220>

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<222> (1)..(96)

21

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 Ser Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
 1 5 10 15
 tct tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
 Ser Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 56
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<400> 56
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 1 5 10 15
 Ser Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 57
 <211> 96
 <212> DNA
 <213> Artificial Sequence

<220>
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<220>
 <221> CDS
 <222> (1)..(96)

<400> 57
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 1 5 10 15

22

tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
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 20 25 30

<210> 58
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<400> 58
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 20 25 30

<210> 59
 <211> 96
 <212> DNA
 <213> Artificial Sequence

<220>
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<220>
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 <222> (1)..(96)

<400> 59
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 20 25 30

<210> 60
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<210> 61
 <211> 96
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 <213> Artificial Sequence

<220>
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<220>
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<400> 61
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 1 5 10 15
 acc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
 Thr Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 62
 <211> 32

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<212> PRT

<213> Artificial Sequence

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Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
 1 5 10 15

Thr Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 63

<211> 96

<212> DNA

<213> Artificial Sequence

<220>

<223> Nucleotide sequence coding for the 1st to the 32nd amino acids of [Ala17]hG-CSF

<220>

<221> CDS

<222> (1)..(96)

<400> 63

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 Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
 1 5 10 15

gcc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
 Ala Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 64

<211> 32

<212> PRT

<213> Artificial Sequence

<400> 64

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Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
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 20 25 30

<210> 65

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<212> DNA

<213> Artificial Sequence

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<220>

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<222> (1)..(96)

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ggc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96

Gly Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 66

<211> 32

<212> PRT

<213> Artificial Sequence

<400> 66

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26

20

25

30

<210> 67
 <211> 96
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Nucleotide sequence coding for the 1st to the 32nd amino acids of [Met2, Val3]hG-CSF

<220>
 <221> CDS
 <222> (1)..(96)

<400> 67
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 1 5 10 15

tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
 Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

<210> 68
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<400> 68
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 20 25 30

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 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Nucleotide sequence coding for the 1st to the 32nd amino acids of [Met2, Val3, Ser17]hG-CSF

<220>
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 1 5 10 15
 tct tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag 96
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 20 25 30

<210> 70
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<400> 70
 Thr Met Val Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
 1 5 10 15
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 20 25 30

<210> 71
 <211> 10
 <212> DNA
 <213> Artificial Sequence

28

<220>

<223> Modified Shine-Dalgarno sequence

<400> 71

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10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00733**A. CLASSIFICATION OF SUBJECT MATTER****IPC7 C12N 15/70**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
NCBI pubmed "GCSF" "signal peptide"**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biotechnol Prog 1995, 11(2):171-7	1-21
A	J Fla Med Assoc 1994, 81(7):467-9	1-21
A	Plasmid 1997;38(3):158-73	1-21
A	Gene 1996, 173(2):271-4	1-21
A	Biochem J 1994, 15;298 Pt 3:719-25	1-21

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 OCTOBER 2000 (31.10.2000)

Date of mailing of the international search report

01 NOVEMBER 2000 (01.11.2000)

Name and mailing address of the ISA/KR

Korean Industrial Property Office
Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
Metropolitan City 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LIM, Hea Joon

Telephone No. 82-42-481-5590

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PCA00729/HMY	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/KR00/00733	International filing date (day/month/year) 07 JULY 2000 (07.07.2000)	Priority date (day/month/year) 08 JULY 1999 (08.07.1999)
International Patent Classification (IPC) or national classification and IPC IPC7 C12N 15/70		
Applicant HANMI PHARM. CO., LTD. et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of <u>3</u> sheets, including this cover sheet. <input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT). These annexes consist of a total of _____ sheets.
3. This report contains indications relating to the following items: I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application

접수
No. 2001.12.14
대한민국
특허법률

Date of submission of the demand 01 FEBRUARY 2001 (01.02.2001)	Date of completion of this report 06 DECEMBER 2001 (06.12.2001)
Name and mailing address of the IPEA/KR Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LIM, Hea Joon Telephone No. 82-42-481-5590

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/KR00/00733

1. Basis of the report

1. With regard to the elements of the international application:*

☒ the international application as originally filed☐ the description:

pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the claims:

pages _____, as originally filed
pages _____, as amended (together with any statement) under Article 19
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the drawings:

pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the sequence listing part of the description:

pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.☒ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☐ The amendments have resulted in the cancellation of:☐ the description, pages _____☐ the claims, Nos. _____☐ the drawings, sheet _____5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed." and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/KR00/00733

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-4, 5-6, 7-18, 19, 20, 21	YES
	Claims		NO
Inventive step (IS)	Claims	1-4, 5-6, 7-18, 19, 20, 21	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-4, 5-6, 7-18, 19, 20, 21	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

1) The following document have been considered for the purpose of this report:

D1=Biotechnol Prog 1995, 11(2):171-7

D2=Plasmid 1997:38(3):158-73

2) Novelty

Claims 1-4, 5-6, 7-18, 19, 20, 21 relate to a modified human granulocyte-colony stimulating factor(hG-CSF), a gene, vector comprising said gene, in which some nucleotide bases coding for the amino acids constituting disulfide bond are substituted with a different bases.

Document D1 and D2 disclosed the methods preparing hG-CSF without modifications, which were different from those of this invention.

Therefore, claims 1-4, 5-6, 7-18, 19, 20, 21 in this invention are considered to be novel.

3) Inventive Step

Claims 1-4, 5-6, 7-18, 19, 20, 21 relate to a modified human granulocyte-colony stimulating factor(hG-CSF), a gene, vector comprising said gene, in which some nucleotide bases coding for the amino acids constituting disulfide bond are substituted with a different bases.

Document D1 and D2 disclosed the methods preparing hG-CSF without modifications, which were different from those of this invention.

The prior arts does not teach or fairly suggest the modification of nucleotide bases coding for the amino acids constituting disulfide bond as a claimed by applicant. The claimed modification or its usefulness for the right folding after the protein production is not taught or suggested by the prior art. The claimed modification contribute for the right structure of the protein is also useful for the transfer of hG-CSF the cytoplasm of the microorganism.

Therefore, claims 1-4, 5-6, 7-18, 19, 20, 21 in this invention discloses modified human granulocyte-colony stimulating factor(hG-CSF), a gene, vector appear to involve an inventive step.

4) Industrial applicability

The subject matter of claims 1-4, 5-6, 7-18, 19, 20, 21 is considered to be industrially applicable.

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

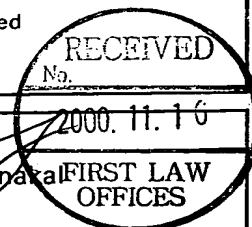
To:

JANG, Seong, Ku
KEC Building
17th floor
275-7, Yangjae-dong
Seocho-gu
Seoul 137-130
RÉPUBLIQUE DE CORÉE

Date of mailing (day/month/year) 31 October 2000 (31.10.00)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference PCA00729/HMY	
International application No. PCT/KR00/00733	International filing date (day/month/year) 07 July 2000 (07.07.00)

1. The following indications appeared on record concerning:		
<input checked="" type="checkbox"/> the applicant	<input type="checkbox"/> the inventor	<input type="checkbox"/> the agent <input type="checkbox"/> the common representative
Name and Address HANMY PHARM. CO., LTD. 893-5, Haejeo-ri Paltan-myeon Hwaseong-gun Kyungki-do 445-910 Republic of Korea	State of Nationality KR	State of Residence KR
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:		
<input type="checkbox"/> the person	<input checked="" type="checkbox"/> the name	<input type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence
Name and Address HANMI PHARM. CO., LTD. 893-5, Haejeo-ri Paltan-myeon Hwaseong-gun Kyungki-do 445-910 Republic of Korea	State of Nationality KR	State of Residence KR
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
3. Further observations, if necessary:		
4. A copy of this notification has been sent to:		
<input checked="" type="checkbox"/> the receiving Office	<input type="checkbox"/> the designated Offices concerned	
<input checked="" type="checkbox"/> the International Searching Authority	<input type="checkbox"/> the elected Offices concerned	
<input type="checkbox"/> the International Preliminary Examining Authority	<input type="checkbox"/> other:	

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Lazar Joseph Panatier
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38



PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference PCA00729/HMY	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/KR00/00733	International filing date (<i>day/month/year</i>) 07 JULY 2000 (07.07.2000)	(Earliest) Priority Date (<i>day/month/year</i>) 08 JULY 1999 (08.07.1999)
Applicant HANMI PHARM. CO., LTD. et al		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
 - ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
 - ☐ contained in the international application in written form.
 - ☒ filed together with the international application in computer readable form.
 - ☐ furnished subsequently to this Authority in written form.
 - ☐ furnished subsequently to this Authority in computer readable form.
 - ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
 - ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant.
- ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant.
- ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawing to be published with the abstract is Figure No. Fig. 1-11

- ☒ as suggested by the applicant.
 - ☐ because the applicant failed to suggest a figure.
 - ☐ because this figure better characterizes the invention.
- ☐ None of the figures.

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C12N 15/70**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI pubmed "GCSF" "signal peptide"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biotechnol Prog 1995, 11(2):171-7	1-21
A	J Fla Med Assoc 1994, 81(7):467-9	1-21
A	Plasmid 1997;38(3):158-73	1-21
A	Gene 1996, 173(2):271-4	1-21
A	Biochem J 1994, 15;298 Pt 3:719-25	1-21

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 OCTOBER 2000 (31.10.2000)

Date of mailing of the international search report

01 NOVEMBER 2000 (01.11.2000)

Name and mailing address of the ISA/KR

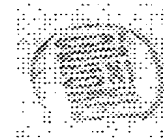
Korean Industrial Property Office
Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
Metropolitan City 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LIM, Hea Joon

Telephone No. 82-42-481-5590




BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

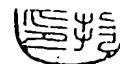
INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd
#893-5 Hajeo-ri Paltan-myun
Hwasung-Kun
Kyonggi-do,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>HM10311</i>	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10154
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March. 24. 1999 (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: April. 6. 1999 <div style="text-align: right; margin-top: 20px;">  </div>

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.




BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd
#893-5 Hajeo-ri Paltan-myun
Hwasung-Kun
Kyonggi-do,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>HM10410</i>	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10151
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March. 24. 1999 (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: April. 6. 1999 


¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd
#893-5 Hajeo-ri Paltan-myun
Hwasung-Kun
Kyonggi-do,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>HM10111</i>	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10152
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March. 24. 1999 (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: April 6. 1999 

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd
#893-5 Hajeo-ri Paltan-myun
Hwasung-Kun
Kyonggi-do,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>HM10510</i>	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10153
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March. 24. 1999 (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authowized official(s) : Date: April. 6. 1999

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.